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Host Response to *Treponema pallidum* in Intradermally-Infected Rabbits: Evidence for Persistence of Infection at Local and Distant Sites

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Intradermal infection of rabbits with *Treponema pallidum* imitiates rapid and active cellular response at the site of injection. During the first 2 weeks following infection, there is a marked increase in the numbers of organisms at the site of infection. Systemic dissemination of treponemes occurs during the early stage of infection, presumably before the immune response is fully mobilized. The mononuclear infiltration, which is apparent at the lesion site one week postinfection, becomes more pronounced at 2 weeks. The infiltrating cells are predominantly T lymphocytes and macrophages. By 4 weeks postinfection, most of the organisms have been cleared from the primary site; however, low numbers of treponemes survive locally and in distant tissues. Thus, whereas infection with T. pallidum appears to activate immune mechanisms which are capable of clearing most of the organisms from the primary lesion, some organisms are able to evade these mechanisms and persist *in vivo*.

The natural course of syphilis has been studied extensively in experimental and clinical situations, but factors determining the ultimate course of infection remain poorly understood. Syphilis is transmitted naturally in humans by direct contact with infectious lesions and may be passed experimentally in rabbits by intratesticular, intravenous, or intradermal injection of virulent *Treponema pallidum*. The rabbit has been the experimental animal of choice for the investigation of early syphilis. In the rabbit, inoculation of *T. pallidum* into the skin produces raised firm dermal lesions, which often progress to ulceration. The time of appearance and duration of dermal lesions is dependent on the number of injected organisms. In addition to multiplication at the site of infection, *T. pallidum* disseminates rapidly to distant tissues where the organisms persist for the lifetime of the animal [1]. A similar sequence of events follows naturally acquired infection in humans. The primary lesion of syphilis, the chancre, usually develops 3–4 weeks after infection. The lesion persists for 1–5 weeks and then heals spontaneously. There is convincing evidence that, during this time, the infectious organisms spread systemically and become the focus of possible later manifestations of syphilis [2].

The mechanism by which infectious treponemes are cleared from the primary site of infection has not yet been clearly defined. We have found that, following intratesticular inoculation of rabbits with *T. pallidum*, a rapid immune response develops. Specifically sensitized lymphocytes may be demonstrated in the spleens of infected animals as early as 3–6 days following infection [3]. Further, an intense mononuclear infiltration, primarily T lymphocytes and macrophages, is observed in the infected testes [4]. The height of this inflammatory response at days 7–10 is followed by the rapid clearance of most *T. pallidum* from the site. The presence of specifically sensitized spleen and lymph node cells within the first week of infection has been reported by others [5,6]. *T. pallidum* reactive lymphocytes in the peripheral blood are not demonstrable until 3 weeks after testicular infection [7].

The present experimental study was designed to examine the host-parasite interactions following intradermal infection in the rabbit. The intradermal route was chosen because it is most analogous to naturally acquired syphilis in humans. The nature of the local immune response and the dissemination of *T. pallidum* from the primary site are described.

MATERIALS AND METHODS

Animals

Sixteen adult male New Zealand white rabbits with nonreactive Venereal Disease Research Laboratory (VDRL) tests were used. They were housed individually at 18–20°C and were given antibiotic-free food and water.

Infection

Treponema pallidum, Nichols strain, was maintained by rabbit testicular passage twice weekly [3]. Treponemes were extracted from infected testicles in 50% heat-inactivated normal rabbit serum saline, tissue debris was removed by centrifugation at low speed, and the organisms were counted by darkfield microscopy. The concentration was adjusted to 2×10^7 motile *T. pallidum* per ml of serum saline. Experimental animals were infected intradermally at 2 or 4 clipped skin sites with 1×10^6 motile *T. pallidum* per site. The rabbits were observed daily for lesion development. At 1, 2, 4 and 6 weeks following inoculation, each of 4 rabbits was sacrificed by exsanguination and injection of a lethal dose of Euthanol 6 (Trico Pharmaceutical Company, San Carlos, CA); dermal lesions, spleen, liver, testes, and popliteal lymph nodes were excised and processed for routine histologic and immunofluorescent examination.

Tissue Specimen Preparation

Tissue specimens (approximately 1 × 1 × 0.3 cm) were placed immediately in cold fixing solution containing 95% ethanol and 1% glacial acetic acid [8]. Following overnight dehydration in absolute ethanol and clearing in xylene, the tissues were embedded in Paraplast Plus Tissue Embedding Medium (Scientific Products, McGaw Park, IL). Adjacent speciments were fixed in 10% formalin for hematoxylin and eosin staining. Serial sections were cut with an American Optical 820 rotary microtome and rehydrated through changes of xylene, 100%, 95% and 70% ethanol, and phosphate buffered saline (PBS) prior to immunofluorescent staining.

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Abbreviations:
ATS: anti-rabbit thymocyte serum
CFA: complete Freund's Adjuvant
FITC: fluorescein isothiocyanate
IFA: incomplete Freund's Adjuvant
PBS: phosphate buffered saline

Serologic Reagents

Human anti-*T. pallidum* serum (Reactive Control Serum) was obtained commercially (Beckman Diagnostics, Fullerton, CA) and was diluted 1/5 in PBS for staining of tissue sections.

Rabbit anti-human globulin was prepared by the intramuscular injection of a rabbit with 0.1 mg DEAE-purified human IgG in complete Freund's Adjuvant (Difco, Detroit, MI) at each of 3 sites, followed at 3 weeks by booster injections in incomplete Freund's Adjuvant (IFA). The animal was bled 1 week following the booster injections; the IgG fraction of the serum was prepared following ammonium sulfate precipitation by DEAE chromatography. Conjugation with fluorescein isothiocyanate (FITC) was performed by standard methods [8,9]; the FITC-IgG protein ratio was approximately 1.7. A dilution of 1/6 (in PBS) was used for tissue labeling.

Goat anti-rabbit thymocyte serum (ATS) was prepared by immunization with 10^9 washed rabbit thymocytes in complete Freund's Adjuvant (CFA). Details of the preparation and extensive characterization of this antiserum have been reported [10–12]. Additionally, the ATS for these studies was absorbed 5 times with cells derived from normal rabbit testes. The specificity of this antiserum has been determined by microcytotoxicity testing and by its effect on *in vitro* immunologic functions; its applicability for immunofluorescent staining has been determined [4,13]. The antiserum was diluted 1/20 in PBS for use in tissue staining.

Goat anti-rabbit immunoglobulin (anti-Ig) was prepared by the intramuscular injection of a goat with 200 μg of DEAE-purified rabbit IgG in CFA at each of 4 sites; booster injections of 50 and 200 μg rabbit IgG in IFA were given at 12 and 20 mo, respectively. This antiserum, which has been characterized by double-diffusion in agar and immunoelectrophoresis, reacts with rabbit IgG and, by virtue of its light chain reactivity, with IgM and IgA. A 1/20 dilution was employed for tissue labeling.

Burro anti-goat IgG was prepared by immunization with DEAE-purified goat IgG in CFA as described above for the preparation of goat anti-rabbit Ig. The IgG fraction of this antiserum was obtained by DEAE chromatography and was labeled with fluorescein isothiocyanate [8,9]; the FITC-IgG protein ratio in the conjugate was 1.7. A dilution of 1/6 was used for tissue labeling.

Immunofluorescent Staining

A double antibody method [13,14] was used to label serial sections of the tissue samples. Deparafinized sections were flooded with the appropriate dilution of the first antiserum (human anti-*T. pallidum*, goat ATS, or anti-Ig on serial sections, respectively) for 30 min at room temperature, and washed 3 times in cold PBS. The appropriate dilution of FITC-conjugated second antiserum (FITC-rabbit anti-human Ig or FITC-burro anti-goat IgG) was applied to the tissue sections and incubation was continued at room temperature in a dark, humidified chamber for 30 min. The slides were washed 3 times with PBS and coverslips were applied with 20% glycerol-PBS mounting medium.

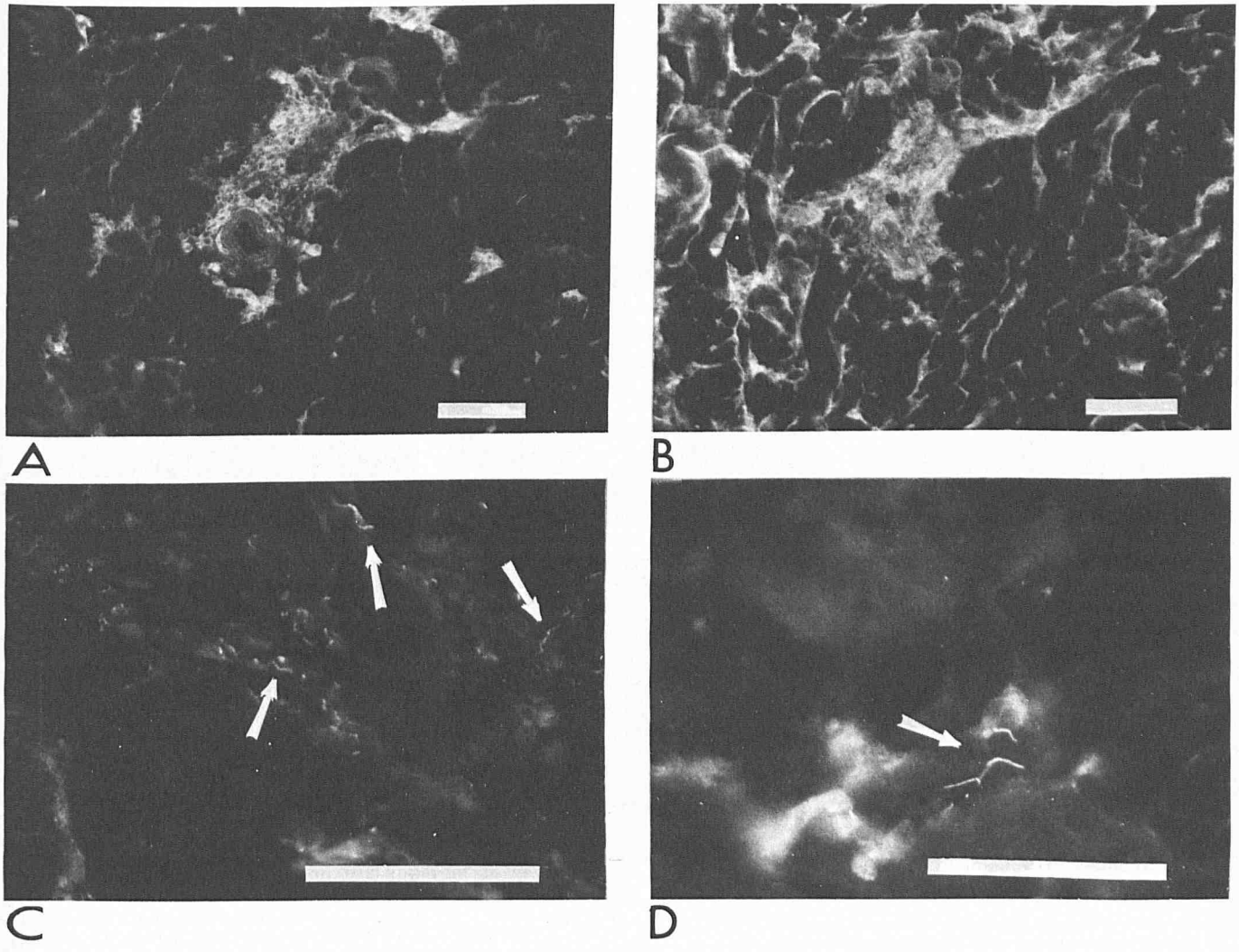

FIG 1. Immunoglobulin, T lymphocyte, and *T. pallidum* localization one week following intradermal injection of 1×10^6 viable *T. pallidum*. *A*, T cells (reduced from × 160). Most of the infiltrating cells stain intensely with ATS and are therefore identified as T lymphocytes. *B*, Ig (B cells) (reduced from × 160). There is diffuse faint staining for Ig which is localized primarily in areas of perivascular mononuclear cell infiltration; only occasional cells stain for Ig. *C*, *D*. *T. pallidum* (reduced from × 400). Numerous *T. pallidum* (marked by arrows) are seen in the areas of perivascular infiltration (*C*) and smaller numbers are scattered throughout the connective tissue of the dermis (*D*). The *white bars* indicate a length of 50 μ.

Controls included the use of normal human or goat serum as the first antibody and the staining of uninfected tissues.

Microscopic Examination

The stained tissue secretions were examined using a Zeiss fluorescent microscope with Phloem illumination. Photomicrographs were taken using Kodak Tri-X film (ASA 400) with exposure times varying from 45 seconds to 2 min.

RESULTS

Gross Appearance

The progression of dermal syphilitic lesions in rabbits has been described in detail elsewhere [1]. The course of infection in the present study was similar to that described by others, with slight temporal variations. Briefly, erythema and induration at the site of injection was first apparent on days 3–4. The raised lesion enlarged and reached a maximum size of approximately 16 mm after 2 weeks. Central ulceration was observed in 100% of the lesions and healing was complete by day 36.

Histologic and Immunofluorescent Examination

Photographs of microscopic sections labeled with antisera to *T. pallidum* and T or B lymphocytes are presented in Fig 1–3. One week after infection there is a slight perivascular and diffuse infiltration of the dermis and subepidermal tissues with mononuclear cells. Most of these cells may be identified by immunofluorescent staining as T lymphocytes (Fig 1*A*). There is a slight diffuse staining with anti-Ig (Fig 1*B*). *T. pallidum* may be identified in the perivascular infiltrates (Fig 1*C*) or in the dermal connective tissue (Fig 1*D*) at that time.

Two weeks post-infection there is a much more pronounced mononuclear cell infiltration (Fig 2). There is more perivascular infiltrate, more inflammatory cells in the upper dermis, and extensive lymphocytic infiltration surrounding the hair follicles. Grossly, at this time the skin lesion is raised, indurated, and often contains a hemorrhagic center. Most of the inflammatory cells are T cells (Fig 2*B*, *D*). Anti-Ig staining is diffuse with only an occasional stained cell seen (Fig 2*C*, *E*). Large numbers of *T. pallidum* are found throughout the dermis with a particular tendency to be localized around hair follicles and in the perivascular infiltrate (Fig 2*F*).

At 4 weeks the inflammation has subsided; some perivascular and perifollicular infiltration remains. ATS and anti-Ig staining is much less intense, with many cells now remaining unstained. At 4 weeks no *T. pallidum* organisms are seen in the tissue sections examined.

Six weeks after injection, further evolution of the inflammatory reaction has occurred (Fig 3). Far fewer mononuclear cells are seen, though some are still scattered throughout the dermis. Subepithelial fibrosis and histiocytic infiltration is prominent. As shown in Fig 3*A*, *B*, immunofluorescence studies reveal that most of the T cell infiltrate is localized around the hair follicles. (This intense and persistent perifollicular inflammation is also seen in humans and may be related to the focal alopecia sometimes seen in late primary and early secondary syphilis). Most of the cells in the subepidermal fibrotic granulomatous zone do not stain with ATS. Immunoglobulin staining is very weak throughout. A few *T. pallidum* organisms are seen in the subepithelial tissues (Fig 3*C*); these organisms appear to be located in fibrous tissue and are not associated with lymphoid cells. Examination of tissues distant from the site of infection also reveal the presence of *T. pallidum*. Most of these are found in the testes (Fig 3*D*), but occasional organisms are also seen in the spleen and lymph node sections as well. Little or no inflam-

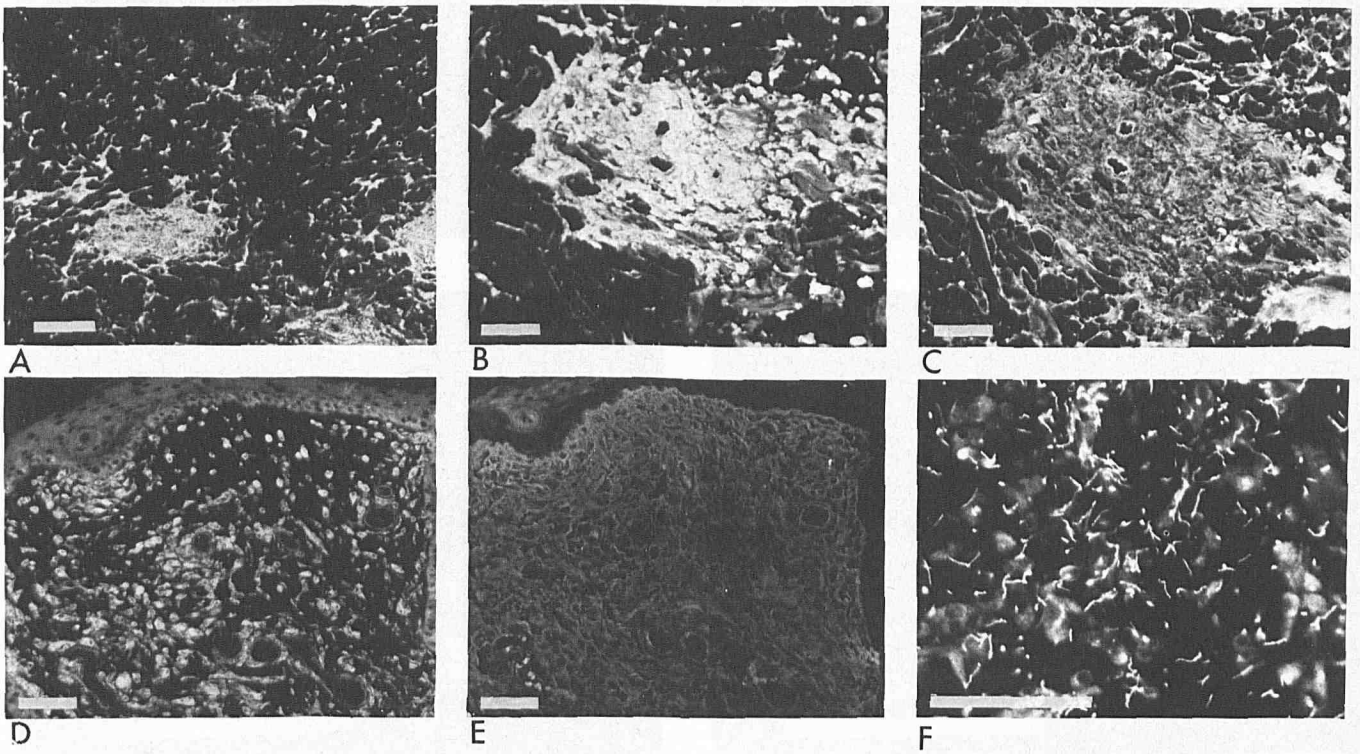

FIG 2. Immunoglobulin (B lymphocytes), T lymphocytes, and *T. pallidum* at the site of inoculation in skin of rabbits infected for 2 weeks. *A*, T cells (reduced from × 63). *B*, T cells (reduced from × 160). *C*, Ig (B cells) (reduced from × 160). *D*, T cells (reduced from × 160). *E*, Ig (B cells) (reduced from × 160). *F*, *T. pallidum* (reduced from ×400). There is now a much more intense mononuclear cell infiltration. T lymphocytes predominate in perivascular infiltrates in the lower dermis (*A*, *B*) and are found diffusely in the upper dermis (*D*). In contrast, there is only faint staining of these areas with anti-Ig (*C* and *E*). Note the intravascular B cells in the lower left corner of *E*. *B* and *C* are serial sections and *D* and *E* are serial sections. Large numbers of *T. pallidum* are seen in the areas of perivascular infiltration (*F*) as well as in the infiltrate surrounding hair follicles. *T. pallidum* also are scattered diffusely throughout the dermis (not shown). The *white bars* indicate a distance of 100 μ for Fig 2*A* and 50 μ for Fig 2 *B–F*.

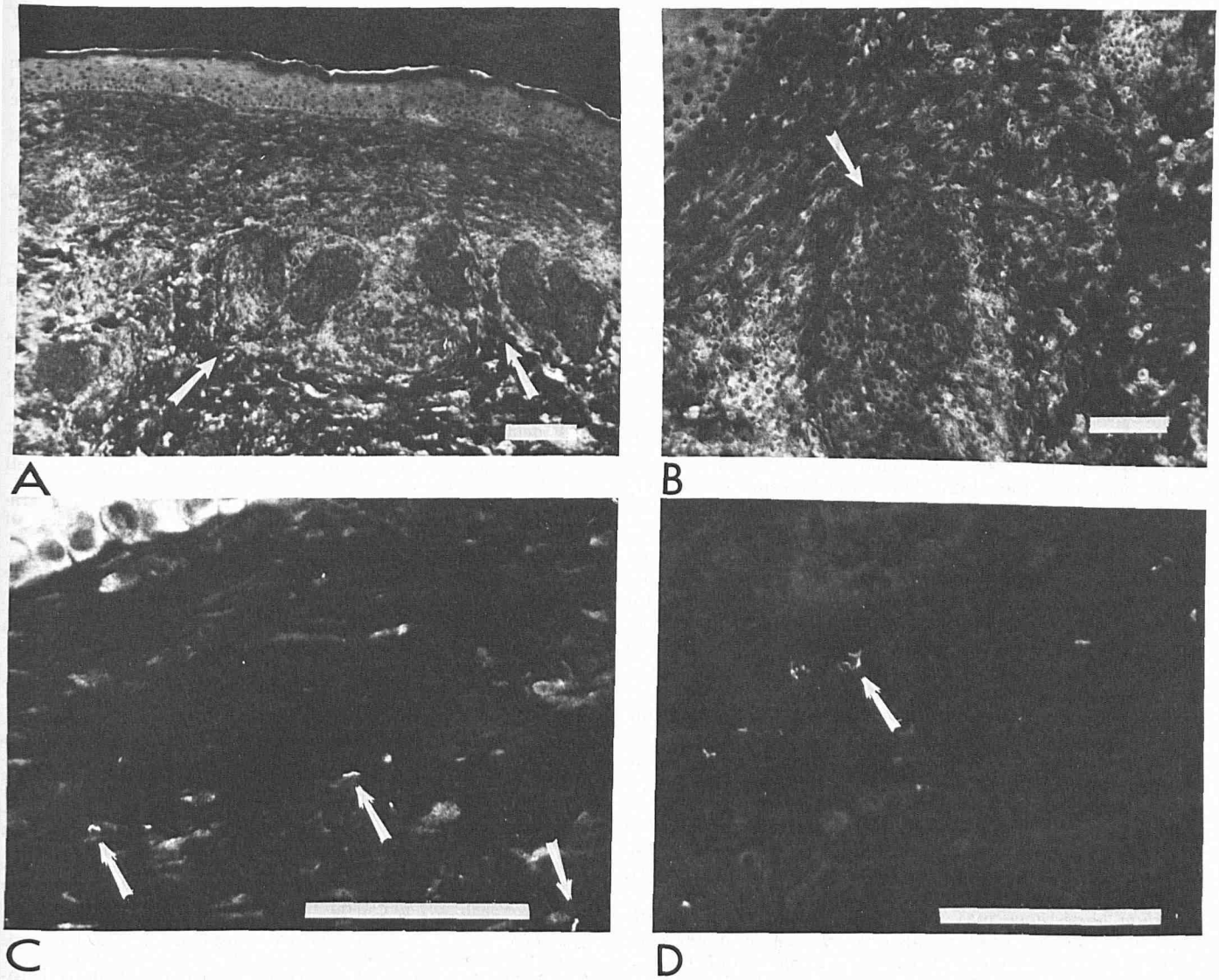

FIG 3. T cells and *T. pallidum* at the site of inoculation in skin and *T. pallidum* in testes of rabbits infected for 6 weeks. *A*, T cells, (reduced from × 63). *B*, T cells (reduced from × 160). *C*, *T. pallidum* in skin (reduced from × 400). *D*, *T. pallidum* in testes (reduced from × 400). Many T cells are still identifiable (*A*), particularly in the perifollicular areas (*B*) (note *arrows*), but the subdermal fibroblastic areas contain few T cells. At this time little or no staining for Ig is observed and only rare B cells are seen (not shown). In the subepithelial granulomatous zone a few *T. pallidum* may still be seen (*C*). Clearly identifiable *T. pallidum* are seen in the testes (*D*). The *white bars* indicate a distance of 100 μ for Fig 3*A* and 50 μ for Fig 3*B–D*.

matory reaction is observed in the testes and no lymphoid cells or immunoglobulin effusion is associated with these organisms. The lymph nodes and spleen are markedly hyperplastic with prominent diffuse cortical and paracortical white pulp expansion.

DISCUSSION

The intradermal infection of adult rabbits with *Treponema pallidum* results in a prompt local infiltration of mononuclear cells accompanied by the clearance of most of the organisms from the primary site of infection. It is apparent, however, that all of the infecting organisms are not destroyed. In addition, examination of distant tissues demonstrates the systemic spread and continued presence of organisms. This is consistent with the observations of others that infection may be transferred from one rabbit to another using lymph node or spleen tissue from rabbits following intratesticular or intradermal infection [1,15,16]. Organisms remain in these tissues even though the lymphocytes from these organs display a specific proliferative response to *T. pallidum* antigens and there is demonstrable serum antibody to *T. pallidum* present [3,5].

The inability of the immune response to eliminate syphilitic infection, thus resulting in the severe late manifestations of the disease, has been the subject of considerable speculation [17]. In recent years, there has existed a growing body of opinion that *T. pallidum* infection results in an impairment or suppression of cellular immunity. This concept has been supported by studies reporting that lymphocytes from infected rabbits [6,18–20] or humans [21–25] display a decreased blastogenic response to nonspecific mitogens or to treponemal antigens. It has been reported that serum or plasma from infected rabbits [7,26,27] and humans [22,28–30] will inhibit the blastogenic response to mitogens.

Studies in our laboratory clearly do not support the conclusion of depressed cellular immunity in *T. pallidum*-infected rabbits. Specifically sensitized lymphocytes may be demonstrated in the spleens of intratesticularly [3] and intradermally (Gamboa D, et al: unpublished data) infected rabbits during

the first week of infection. An extensive T cell infiltration occurs at the site of primary infection and is accompanied by the apparent elimination of most of the offending organisms. There is also a marked hyperplasia of draining lymph nodes and the spleen with extensive T cell proliferation in paracortical and periarteriolar zones [3]. These findings alone are convincing evidence that an appropriate immune response takes place. In addition, we have not been able to confirm reports that infection of rabbits with syphilis causes a suppression of lymphocyte responsiveness *in vitro* or that serum from infected rabbits significantly suppresses the *in vitro* response of lymphocytes to Concanavalin A. (Lukehart SA, Baker-Zander SA, Sell S: to be published). *T. pallidum*-infected rabbits respond by formation of humoral antibody and lymphocyte reactivity to *T. pallidum* antigens of the magnitude expected in an immunocompetent animal. There is no evidence of impaired induction of T or B cell immunity to *T. pallidum*; therefore, another explanation for the persistence of *T. pallidum in vivo* must be sought.

There are numerous examples of other infectious organisms which survive despite the active immune defenses of the host. Bloom has recently reviewed mechanisms by which parasites escape immune attack [32]. These mechanisms include (1) adaptive phenotypic variation in antigens expressed by the organisms, (2) mimicry of the host by acquisition of host antigens, (3) adaptation to survival inside macrophages by altering macrophage metabolism or developing resistance to lysosomal enzymes, (4) direct infection of immune lymphocytes, or (5) suppression of the immune response. As stated above, most attention with regard to syphilis infection has recently been focused on the last mechanism. However, on the basis of the recent observations reported in this paper and elsewhere, we suggest that more attention should be given to other possible mechanisms of persistence.

In addition to the mechanisms of escape described by Bloom, other possible mechanisms may be operative in syphilis. Organisms may persist in locations which are normally inaccessible to immune attack. Indeed, common sites of late granulomatous lesions include the brain and the wall of the aorta, which are relatively avascular and not accessable to immune products. The lack of cellular infiltration at disseminated sites of *T. pallidum* persistence may indicate weak antigenicity of the infecting organisms. Large numbers of treponemes may simply be necessary to provide a sufficient antigenic stimulus for an inflammatory response. Indeed, the time of cellular infiltration following intradermal infection in the rabbit is dependent upon the number of injected organisms. Alternatively, antigenic sites on the bacteria may be masked by host or parasite-derived products. A mucopolysaccharide "slime coat" is associated with *T. pallidum in vivo*. This layer may provide protection against the activities of bactericidal antibody and/or phagocytic cells [32–34]. Finally, in addition to the protective role of antibody, specific antibody or antigen-antibody complexes may produce a blocking effect [35–37] which could inhibit cellular defense mechanisms. Clearly, the host-parasite relationship in syphilis is an area of study which deserves more critical attention.

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Announcement

In 1981, the Certifying Examination of the American Board of Dermatology will be held on November 1 and 2 in Chicago, Illinois. The deadline for receipt of applications is May 1, 1981.

The Dermatopathology special competence examination will be held in Chicago, Illinois on November 3; the deadline for receipt of applications is July 1, 1981.

For further information on any of these examinations, please contact: Clarence S. Livingood, M.D., Executive Director, American Board of Dermatology, Henry Ford Hospital, Detroit, MI 48202.